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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A61K 9/127, 9/133	A1	(11) International Publication Number: WO 98/22092
		(43) International Publication Date: 28 May 1998 (28.05.98)

(21) International Application Number: PCT/US97/21352

(22) International Filing Date: 20 November 1997 (20.11.97)

(30) Priority Data:
60/031,636 22 November 1996 (22.11.96) US

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(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: TRANSPORT OF LIPOSOMES ACROSS THE BLOOD-BRAIN BARRIER

(57) Abstract

A brain-specific liposome targeting vehicle for transporting neuropharmaceutical agents across the blood-brain barrier (BBB). The targeting vehicle includes a liposome which is sterically stabilized by attaching ligands to the surface of the liposome. The targeting vehicle further includes blood-barrier transport agents which are attached to the tail portion of the stabilizing ligands which extend outward from the liposome surface. The blood-barrier transport agents are capable of transporting the entire liposome targeting vehicle across the blood-brain barrier. Monoclonal antibodies which undergo receptor-mediated transcytosis across the BBB are featured as useful blood-barrier transport agents. The resulting brain-specific immunoliposomes are well-suited for delivering a wide variety of neuropharmaceutical agents across the blood-brain barrier.

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TRANSPORT OF LIPOSOMES ACROSS THE BLOOD-BRAIN BARRIER

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates generally to delivering neuropharmaceutical agents to the brain for both diagnosis and/or treatment of diseases which are specific to the brain. More particularly, the present invention involves the use of a brain-specific liposome targeting vehicle which is capable of transporting neuropharmaceutical agents across the blood-brain barrier (BBB) and into the brain.

2. Description of the Related Art

The publications and other reference materials referred to herein to describe the background of the invention and to provide additional detail regarding its practice are hereby incorporated by reference. For convenience, the reference materials are listed numerically in the body of the specification and identified in the appended bibliography.

The vertebrate brain has a unique capillary system which is unlike that in any other organ in the body. The unique capillary system has morphologic characteristics which make up the blood-brain barrier (BBB). The blood-brain barrier acts as a systemwide cellular membrane which separates the brain interstitial space from the blood.

The unique morphologic characteristics of the brain capillaries which make up the BBB are: (a) epithelial-like high resistance tight junctions which literally cement all endothelia of brain capillaries together, and (b) scanty pinocytosis or transendothelial channels, which are abundant in endothelia of peripheral organs. Due to the unique characteristics of the blood-brain barrier, hydrophilic drugs and peptides that readily gain access to other tissues in the body are barred from entry into the brain or their rates of entry are very low.

Various strategies have been developed for introducing those drugs into the brain which otherwise would not cross the blood-brain barrier. The most widely

used strategies involve invasive procedures where the drug is delivered directly into the brain. The most common procedure is the implantation of a catheter into the ventricular system to bypass the blood-brain barrier and deliver the drug directly to the brain. These procedures have been used in the treatment of brain diseases which have a predilection for the meninges, e.g., leukemic involvement of the brain.

Although invasive procedures for the direct delivery of drugs to the brain ventricles have been practiced, their use has not been entirely successful because they only distribute the drug to superficial areas of the brain tissues, and not to the structures deep within the brain. Further, the invasive procedures are potentially harmful to the patient.

Other approaches to circumventing the blood-brain barrier utilize pharmacologic-based procedures involving drug latentiation or the conversion of hydrophilic drugs into lipid-soluble drugs. The majority of the latentiation approaches involve blocking the hydroxyl, carboxyl and primary amine groups on the drug to make it more lipid-soluble and therefore more easily transported across the blood-brain barrier. However, the disadvantage of this approach is that the enhanced lipid solubility of the lipidized molecule also increases uptake of the drug by non-brain tissues. This decreased availability of the drug in the blood stream offsets any increased BBB penetration caused by the lipidization modification.

Another approach to circumventing the blood-brain barrier involves the intra-arterial infusion of hypertonic substances which transiently open the blood-brain barrier to allow passage of hydrophilic drugs. However, hypertonic substances are potentially toxic and may damage the blood-brain barrier.

One approach which has shown promise involves attaching drugs to a transport vector to form a chimeric compound which is capable of crossing the BBB. The transport vectors have been peptides or modified proteins that undergo receptor-mediated transcytosis through the BBB (38).

Immunoliposomes (antibody-directed liposomes) have been recognized as a promising tool for the site-specific delivery of drugs and diagnostic agents. However, the *in vivo* use of classical immunoliposomes is hampered by the very rapid clearance of immunoliposomes from the circulation by the reticuloendothelial system (1, 2). Avoidance of this obstacle is possible if gangliosides (3) or PEG-

derivatized lipids (4) are inserted within the bilayer of conventional liposomes, as these modifications prolong considerably the liposome half-life in the circulation. Liposomes coated with the inert and biocompatible polymer PEG are widely used and are often referred to as "sterically stabilized" or "stealth liposomes" (5). PEG coating is believed to prevent recognition of liposomes by macrophages due to reduced binding of plasma proteins (4, 6). Unfortunately, it has been difficult to combine steric stabilization of liposomes with efficient immunotargeting. PEG coating of liposomes can create steric hindrances for antibody-target interaction (7, 8). It has therefore been proposed to attach a cell-specific ligand to the distal end of a few lipid-conjugated PEG molecules rather than conjugate the ligand to a lipid headgroup on the surface of a PEG-conjugated liposome. This has been done recently with folic acid (9) and monoclonal antibodies (10-13 and 37) to target liposomes to cells in tissue culture and organs *in vivo*.

Although immunoliposomes have shown promise in general, they have not been proposed for use in delivering drugs to the brain because native liposomes do not cross the BBB. Liposomes, even small unilamelar vesicles, are simply too large to cross the BBB (14-16). Accordingly, the use of immunoliposomes has been limited to non-brain targeting of drugs.

In view of the above, there presently is a need to provide improved substances and methods for delivering neuropharmaceutical agents across the blood-brain barrier and into the brain. It is desirable that such improved substances and methods provide for uniform introduction of the neuropharmaceutical agent throughout the brain and present as little risk to the patient as possible.

SUMMARY OF THE INVENTION

The present invention is based on the discovery that a liposome can be altered in such a way as to form a brain-specific targeting vehicle which is capable of delivering neuropharmaceutical agents across the blood-brain barrier. The present involves two features which, when combined together, provide an especially useful way to deliver neuropharmaceutical agents to the brain. The first feature involves sterically stabilizing a liposome by attaching a ligand to the surface of the liposome. This feature helps to prolong the life of the liposome once it is introduced *in vivo*.

The second feature involves attaching a blood-barrier transport agent to the exposed tail portion of the ligands. In accordance with the present invention, it was surprisingly discovered that blood-barrier transport agents which were attached to the liposome via the stabilizing ligand were capable of carrying or transporting the stabilized liposome across the blood-brain barrier. Altering liposomes to include the above two mentioned features provides a liposome targeting vehicle in which a wide variety of neuropharmaceutical agents can be encapsulated for eventual delivery across the blood-brain barrier.

In accordance with the present invention, a brain-specific liposome targeting vehicle is provided which is capable of transporting neuropharmaceutical agents across the blood-brain barrier. The liposome targeting vehicle includes a liposome which is a lipid vesicle that has an exterior surface and interior surface. The interior surface of the vesicle defines a compartment in which neuropharmaceutical agents are encapsulated. At least one ligand, which includes a head and a tail portion, is attached to the exterior surface of the liposome vesicle by way of the ligand head portion. Blood-barrier transport agents are attached to the tail portion of the ligands. The transport agent is a peptide, protein or antibody which is capable of transporting itself and the conjugated liposome across the blood-brain brain barrier by receptor-mediated transcytosis. As a final element, the targeting vehicle includes a neuropharmaceutical agent located within the liposome compartment.

As one aspect of the present invention, it was discovered that from 10 to 40 blood-barrier transport agents should be attached to each liposome to obtain maximum liposome transport across the blood-brain barrier. Liposome targeting vehicles having about 30 transport agents attached to the stabilizing ligands were found to be optimum.

The brain-specific targeting vehicle of the present invention is well-suited for use in a wide variety of situations for delivering neuropharmaceutical agents to the brain via intravenous injection or other blood-based administration route. Diseases in which the targeting vehicle may be used include Alzheimer's disease, cerebral AIDS, brain cancer, epilepsy, neurodegenerative diseases, stroke, drug abuse, multiple sclerosis, anxiety, and other neurologic or mental disorders.

The above discussed and many other features and attendant advantages will become better understood by reference to the following detailed description when taken in conjunction with the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic diagram showing the preparation of a preferred exemplary brain-specific liposome targeting vehicle in accordance with the present invention. The schematic diagram shows the coupling of the thiolated monoclonal antibody OX26 (OX26-SH) with sterically stabilized liposomes containing DSPE-PEG-maleimide; DSPE = distearoyl phosphatidyl ethanolamine; PEG = polyethyleneglycol.

FIG. 2 is a graph showing the size distribution of sterically stabilized liposomes prepared by rapid extrusion.

FIG. 3 shows the elution profile of the separation of [^{3}H]daunomycin-loaded immunoliposomes from unencapsulated [^{3}H]daunomycin and unbound ^{125}I-labeled mAb OX26 by gel filtration; mAb = monoclonal antibody.

FIG. 4 show the percent injected dose per milliliter (%ID/ml) remaining in the blood stream after given times for free daunomycin, unaltered liposomes, liposomes which have been sterically stabilized with polyethylene glycol ligands (PEG-liposomes) and PEG-liposomes conjugated with OX26 monoclonal antibodies; OX_{29} = liposomes with 29 OX26 mAb molecules attached.

FIG. 5 shows the %ID/ml for targeting vehicles in accordance with the present invention where the number of monoclonal antibodies per liposome are 3, 21 and 197. The results for PEG-liposomes without a conjugated antibodies are also shown for comparison.

Fig 6. depicts the results of tests showing the brain volume of distribution (V_d) for free daunomycin (D), daunomycin incorporated in unaltered liposomes (L),

brain-specific liposomes in accordance with the present invention where 29 OX26 antibodies are attached to each liposome (29) and PEG-conjugated liposomes without conjugated antibodies (0). The brain volume of distribution was measured 60 minutes after intravenous injection.

FIG. 7 depicts the results of tests showing permeability-surface area (PS) product (PS) 60 minutes after intravenous injection for the same compounds shown in FIG. 6.

FIG. 8 depicts the results of tests showing area under the plasma concentration curve (AUC) 60 minutes after intravenous injection for the same compounds shown in FIG. 6.

FIG. 9 depicts the results of tests showing the percent of injected dose per gram of brain tissue 60 minutes after intravenous injection for the same compounds shown in FIG. 6.

FIG. 10 depicts the results of tests showing the brain volume of distribution (60 minutes after intravenous injection) for liposome targeting vehicles in accordance with the present invention which have 3, 21, 29 and 197 antibodies, respectively, attached to each liposome. The results for a PEG-protected liposome having no attached antibodies are also shown for comparison.

FIG. 11 depicts the results of tests showing the permeability-surface area product for the same liposome targeting vehicles shown in FIG. 10 (60 minutes after intravenous injection).

FIG. 12 depicts the results of tests showing percent injected dose per gram of brain tissue for the same liposome targeting vehicles shown in FIG. 10 (60 minutes after intravenous injection).

FIG. 13 depicts the results of tests showing AUC for the same compounds shown in FIG. 10 (60 minutes after intravenous injection).

FIG. 14 depicts the results of tests where a liposome targeting vehicle in accordance with the present invention having 30 antibodies per liposome was measured for brain V_d at times ranging up to 24 hours after intravenous injection.

DETAILED DESCRIPTION OF THE INVENTION

The brain-specific liposome targeting vehicles of the present invention may be used to transport a wide variety of neuropharmaceutical agents across the blood-brain barrier (BBB). Neuropharmaceuticals include both neurodiagnostic and neurotherapeutic agents. Exemplary neurodiagnostic agents include Congo Red for Alzheimer's disease, or EGF analogues for brain tumors. Exemplary neuro-therapeutic agents include thyrotropin releasing hormone (TRH) which is used to treat spinal cord injury and Lou Gehrig's disease; vasopressin which is used to treat amnesia; alpha interferon which is used to treat multiple sclerosis; somatostatin which is used to treat Alzheimer's disease; endorphin which is used to treat pain; L-methionyl (sulfone)-L-glutamyl-L-histidyl-L-phenylalanyl-D-phenylalanine (an analog of adrenocorticotrophic hormone (ACTH-4-9) which is used to treat epilepsy; muramyl dipeptide which is used to treat insomnia; daunomycin which is an antineoplastic agent; and azidothymidine for cerebral AIDS, NMDA receptor blockers for stroke, amphotericin for brain infections; opioid peptide analogues for drug abuse; corticotropin releasing hormone (CRH) analogues for eating disorders. All of the above neuropharmaceutical agents are available commercially or they may be isolated from natural sources by well-known techniques. The above-listed neuropharmaceutical agents are exemplary of the types of agents which may be encapsulated in the liposome targeting vehicles of the present invention. Other neuropharmaceutical agents may be used provided that they are amenable to encapsulation in liposome.

The brain-specific targeting vehicles include a liposome which is sterically stabilized by attaching stabilizing ligands to the surface of the liposome. Blood-barrier transport agents are then attached to the tails of the ligands to provide a

targeting vehicle which is capable of crossing the BBB and delivering encapsulated neuropharmaceutical agents to the brain. The basic procedure for forming the targeting vehicle is set forth in FIG. 1. The ligand is polyethylene glycol (PEG) and the transport agent is the monoclonal antibody OX26 which is attached to the PEG ligands via a sulfur linkage.

The liposomes which can be used to make brain-specific targeting vehicles include both unilamelar and multilamelar vesicles. Unilamelar vesicles are preferred. The liposomes may be made from any of the natural or synthetic lipids which have been used to form liposomes. Exemplary vesicle forming lipids include phosphatidylcholine, phosphatidic acid, phosphatidylglycerol, phosphatidylinositol, sphingomyelin, dimyristoylphosphatidylcholine, dipalmitoylphosphatidylcholine, phosphatidylethanolamine and distearyl phosphatidylethanolamine. Distearyl phosphatidylethanolamine is a preferred liposome forming lipid. Any other liposome forming lipid may be used provided that it is capable of forming a liposome which can encapsulate the desired neuropharmaceutical agent and which can be sterically stabilized with a stabilizing ligand and made BBB permeable by attachment to an appropriate blood-barrier transport agent.

The liposomes are prepared according to well-known conventional procedures. The liposomes should have diameters which are less than 100 microns. Liposomes with larger diameters would be difficult to transport across the BBB. Liposomes having diameters in the range of about 40 microns to about 80 microns are preferred. The procedures for making liposomes are well-known and basically involve mixing lipids of defined composition in appropriate relative amounts, and then forming nanoparticle-like structures with sonication, extrusion, or freeze-thawing methods.

Other nanoparticles having diameters of less than 100 microns may be used in place of liposomes. When non-liposome nanoparticles are used, the neuropharmaceutical agent is usually attached to the surface of the nanoparticle instead of being encapsulated.

The stabilizing ligands which are attached to the liposome surface can be any ligand which provides steric stabilization of the liposome. Suitable ligands include gangliosides and polymers. Polymers such as polyethylene glycol (PEG) of

molecular weight of 2000-5000 daltons are preferred ligands. The polymer ligands should have molecular weights of between 200 daltons and 5000 daltons. PEG ligands having molecular weights of between 2000 daltons and 5000 daltons are preferred ligands. PEG ligands having a molecular weight of about 2000 daltons are particularly preferred. The ligands are attached to the liposome surface using known conventional procedures. The procedures basically involve incorporation of the ligand into a lipid moiety, e.g., DSPE, that is to be subsequently incorporated into the liposome or nanoparticle. The procedure for attaching PEG ligands to liposomes is known as "pegylation". The resulting sterically stabilized liposome is referred to as a "pegylated" liposome. The portion of PEG or other ligand which is attached directly to the liposome surface is the head of the ligand. The opposite end of the ligand which is displaced away from the liposome surface is known as the tail of the ligand. The number of ligands attached to each liposome is preferably in the range of between about 10 and 100. Alternatively, gangliosides may be used as ligands. Glycerophosphatidylinositol (GPI) may be covalently attached to the BBB transport agent in biological expression cultures.

After attachment of the stabilizing ligand, a blood brain-barrier transport agent is attached to the tail of the ligand. The transport agent must be capable of transporting the stabilized liposome across the BBB. Suitable transport agents include peptides, proteins and antibodies which are capable of receptor-mediated or absorptive-mediated transcytosis across the BBB. Monoclonal antibodies are the preferred transport agent. Exemplary peptides which may be used as transport agents include insulin, transferrin, insulin-like growth factor I (IGF-I), insulin-like growth factor II (IGF-II), leptin, basic albumin and prolactin. These peptides have previously been used as transport agents to carry hydrophilic peptides across the BBB (38). Exemplary monoclonal antibodies include antibodies against the transferrin, insulin, IGF, lectin, or prolactin receptor. Such antibodies may be prepared with standard hybridoma technology and immunization of the mice with either purified receptor protein or synthetic peptides incorporating specific amino acid sequences with extracellular-projecting domains of the receptor. The OX26 antibody may be obtained from Dr. Arthur Like (Worcester, MA) and the 83-14 antibody may be obtained from Dr. Kenneth Siddle (Cambridge, England).

Any of the antibodies which have been shown to undergo receptor-mediated transcytosis through the BBB may be used as a transport agent (17, 18). OX26 is a preferred monoclonal antibody for studies in rats. It is an antibody to the transferrin receptor (19) which is abundant on brain microvascular endothelium (21, 22). The 83-14 mAb to the human insulin receptor is the preferred transport agent for humans or primates (39).

When monoclonal antibodies are used as the blood-barrier transport agent, the liposome is referred to as an "immunoliposome." Monoclonal antibodies to the insulin receptor may also be used. For example, monoclonal antibody 83-14 is an antibody to the human insulin receptor which may be used as a transport agent (39).

The brain-barrier transport peptide or monoclonal antibody is attached by conjugation to the tail of the ligand. The conjugation may be carried out using bifunctional reagents which are capable of reacting with the transport agent and the ligand tail to form a bridge between the two. A preferred method of conjugation involves thiolation of the peptide or antibody to form a sulfur bridge or linkage between the ligand and the peptide or antibody. Other conjugation agents may be used. However, thiolation is preferred because it does not cause any denaturization of the peptide or antibody. Exemplary conjugation agents include those that form thiolether, amide, ester, or disulfide linkages. The conjugation of the blood-transport agent to the ligand is carried out according to known procedures. These procedures basically involve using standard chemical crosslinkers such as Traut's reagent, MBS, SPOP. Alternatively, a glycerophosphoinositol (GPI) moiety may be attached to the BBB transport agent for direct incorporation within the liposome. It is preferred that the conjugation process be controlled so that from 10 to 40 transport agents are attached to each liposome. It was discovered that too few transport agents resulted in low delivery levels and that too many transport agents also caused decreases in delivery levels. Optimum delivery of liposomes across the BBB is achieved when from 25 to 35 transport agents are attached to each liposome via the ligands.

Exemplary brain-specific liposome targeting vehicles in accordance with the present invention are as follows: radiolabeled amyloid imaging agent incorporated

in 83-14 mAb pegylated immunoliposomes; AIDS triple therapy cocktail incorporated within cationized albumin pegylated liposome.

The liposome targeting vehicles can be introduced into the body by any conventional procedure which delivers the targeting vehicles to the blood stream. Intravenous injection is preferred. Parenteral injection and intranasal inhalation is also suitable. The liposome targeting vehicles are combined with a compatible pharmaceutical carrier and injected or inhaled in accordance with well-known conventional procedures for administering liposomes to the blood stream. Suitable carrier solutions include sterile saline which includes common bacteriostatic agents. The concentration of liposome targeting vehicles in the carrier will vary depending upon the specific disease being treated or the specific diagnostic purpose. The dosage levels will also vary depending upon the diagnostic or therapeutic purpose. In general, the dosage and concentration levels will correspond to the accepted and established dosages for the particular neuropharmaceutical agent which is being used.

Examples of practice are as follows:

Materials. Cholesterol was obtained from Sigma Chemical Co. (St. Louis, MO). Distearoylphosphatidylcholine and distearoylphosphatidylethanolamine (DSPE) were from Avanti Polar Lipids (Alabaster, AL). DSPE-PEG was purchased from Shearwater Polymers (Huntsville, AL). DSPE-PEG-maleimide was custom-synthesized by Shearwater Polymers. DSPE-PEG-maleimide was prepared by derivatization of DSPE-PEG. The efficiency of this reaction was 84%, as determined by NMR analysis. PEG^{2000} was used in all PEG-containing compounds. [^{3}H]Daunomycin was obtained from DuPont/NEN (Boston, MA). 2-Iminothiolane (Traut's reagent) and the bicinchoninic acid protein assay kit (used with bovine serum albumin as a standard) were obtained from Pierce Chemical Co. (Rockford, IL). Sephadex G-25, Sepharose CL-4B, and protein G Sepharose were obtained from Pharmacia (Piscataway, NJ). Mouse myeloma ascites IgG_{2a} (κ) was from Cappel (Durham, NC). Centriprep-30 (molecular weight cut-off: 30,000) concentrators were from Amicon (Beverly, MA). Male Sprague-Dawley rats (weighing 260-290g) were obtained from Harlan-Sprague-Dawley (Indianapolis, IN). All other chemicals were of analytical grade.

Purification and Thiolation of Antibodies. The anti-rat transferrin receptor OX26 mAb (19) was harvested from cell culture supernatants of the OX26 hybridoma cell line as described (22). OX26 as well as the control IgG_{2a} antibody were purified by protein G Sepharose affinity chromatography (23). OX26 mAb was iodinated to a specific activity of 10 μCi/μg (1 Ci = 37 Gbq) using [^{125}I]iodine and chloramine T, as described previously (24). The radiolabeled protein was purified by Sephadex G-25 gel filtration chromatography and was >96% TCA-precipitable. [^{125}I]OX26 or IgG_{2a} were thiolated using 2-iminothiolane (Traut's reagent; ref. 25). mAb was dissolved in 0.15 M Na-borate buffer/0.1 mM EDTA, pH 8.5 followed by the addition of Traut's reagent. After incubation for 60 minutes at room temperature, mAb solutions were concentrated and the buffer exchanged with 0.1 M Na-phosphate (pH 8.0) using a Centriprep-30 concentrator (Amicon). Thiolated mAb was immediately used for conjugation with liposomes (*see* below). Ellmann's reagent (26) was used to determine the number of sulhydryl groups added by thiolation to mAb. Using a mAb/Traut's ratio of 1:40 mol/mol), an average of one primary amine per mAb was thiolated.

Liposome Preparation. Distearoylphosphatidylcholine (5.2 μmol), cholesterol (4.5 μmol), DSPE (0.3 μmol), and, for the preparation of immunoliposomes, linker lipid (DSPE-PEG-maleimide, 0.015 μmol) were dissolved in chloroform/methanol [2:1 (vol/vol)]. For the synthesis of PEG-liposomes and immunoliposomes, DSPE was substituted by DSPE-PEG. A lipid film was prepared by vacuum evaporation using a Speedvac concentrator (Savant) for 70 minutes. Dried lipid films were hydrated at 65°C in 0.3 M citrate (pH 4.0), such that a final lipid concentration of 10 mM was achieved. Lipids were subjected to five freeze-thaw cycles followed by extrusion (4 times) at room temperature through a 100-nm pore size polycarbonate membrane employing an extruder (Avestin, Ottawa, Canada). Extrusion was repeated 9 times using a 50-nm polycarbonate membrane. Mean vesicle diameters were determined by quasielastic light scattering using a Microtrac Ultrafine Particle Analyzer (Leeds-Northrup, St. Petersburg, FL).

Daunomycin Loading and Antibody Conjugation. Loading of liposomes with [^{3}H]daunomycin was done via a pH gradient (27). By addition of NaOH to liposomes (10 μmol of lipid), the pH of the external buffer was raised to pH 7.8.

[^{3}H]Daunomycin was added and the incubation mix was incubated for 10 minutes at 60°C. Loaded liposomes were either used for coupling with mAb or external buffer was exchanged by passing the liposomes over a Sephadex G-25 column and eluting with 0.001 M PBS (0.001 M Na-phosphate/0.15 M NaCl, pH 7.4). For antibody conjugation, thiolated antibody was incubated with liposomes containing DSPE-PEG-maleimide overnight at room temperature. Buffer was exchanged by applying the reaction mix to a 1.6 x 18 cm Sephadex CL-4B column and eluting with 0.001 M PBS. Aliquots of column eluates were analyzed by scintillation counting. Efficacy of entrapment of daunomycin and efficiency of OX26 coupling was determined by analysis of the column elution profiles. In the case where no ^{125}I-labeled antibody was available (control IgG_{2a} mAb), the amount of liposome-conjugated protein was determined by a protein assay as described (28). The number (n) of OX26 molecules attached per liposome are designated as $OX26_n$.

Pharmacokinetics and Brain Delivery of Immunoliposomes. Pharmacokinetic experiments were performed as described (29). Rats were anesthetized with 100 mg of ketamine and 2 mg of xylazine per kg of body weight intraperitoneally. The left femoral vein was cannulated and injected with 0.001 M PBS containing 4 μCi of free [^{3}H]daunomycin, [^{3}H]daunomycin-loaded liposomes, or immunoliposomes. The injected dose of mAb and lipid was always ≤ 1 μmol per rat, respectively. Blood samples were collected via a cannula implanted in the left femoral artery at 0.25, 1, 2, 5, 15, 30, and 60 minutes after intravenous injection of the isotope. The blood volume was replaced with an equal volume of saline. After 60 minutes, the animals were decapitated for removal of the brain. For some experiments, animals were sacrificed 6 hours or 24 hours after intravenous injection. In this case, animals were allowed to recover after surgery and terminal blood only was sampled. The plasma and organ samples were solubilized with Soluene-350 (Packard) and neutralized with glacial acetic acid before liquid scintillation counting. Pharmacokinetic parameters were calculated by fitting plasma radioactivity data to a biexponential equation—$A(t) = A_1 = e^{-k1t} + A_2 = e^{-k2t}$, where $A(t)$ = %ID per ml of plasma ^{3}H radioactivity (%ID, percent injected dose). The biexponential equation was fit to plasma data using a nonlinear regression analysis. The pharmacokinetic parameters, such as plasma clearance (Cl), the initial plasma volume (V_C), steady-

state volume of distribution (V_{ss}) and steady-state area under the plasma concentration curve (AUC_0) were determined from A_1, A_2, k_1, and k_2. The brain volume of distribution (V_D) of the [^{3}H]sample at a given timepoint after i.v. injection was determined from the ratio of disintegrations/minute (dpm) per gram of tissue divided by the dpm/μl of terminal plasma. The brain permeability surface area (PS) product was determined as follows:

$$PS = \frac{[V_D - V_0]Cp(T)}{\int_0^t Cp(t)\,dt},$$

where $Cp(T)$ = the terminal plasma concentration and V_0 = the brain volume distribution of a plasma volume marker. The brain volume of distribution of PEG-liposomes is 7.48 ± 0.34 μl/g (23). The brain V_D of the PEG-liposomes were therefore used as a plasma volume marker (V_0) for the calculation of brain PS products. Brain uptake was expressed as %ID per g of brain and was calculated from: $\%ID/g(t) = PS \times AUC_0$.

Daunomycin Octanol/Aqueous Partition Coefficient. The 1-octanol/buffer partition coefficient (P) was determined for [^{3}H]daunomycin as described previously (17) and was 0.40 ± 0.04 (mean ± SEM, n = 3), which yielded a logarithmic P = -0.40.

The liposomes prepared by the above procedure had an average diameter of 85 nm with a sharp distribution of size (80% of the liposomes had a size between 65 and 115 nm; FIG. 2). Using a pH-shift method, loading of liposomes with [^{3}H]daunomycin was achieved with high and reproducible yield (89.9 ± 1.1%, value is mean ± SEM, n = 7). Loading was not affected by lipid composition of the vesicles or the lipid to daunomycin molar ratio (range of ratios tested was from 16:1 to 2500:1). For the synthesis of immunoliposomes, the linker lipid DSPE-PEG-maleimide was incorporated in liposomes. Daunomycin-loaded vesicles were allowed to react with thiolated antibody (FIG. 1). mAb 0X26 was thiolated using 2-iminothiolane (Traut's reagent). The ratio of Traut's reagent and mAb was adjusted to yield an average of one thiolated primary amine per mAb. It has been shown previously (30) that thiolation of OX26 does not interfere with its target

recognition. Since the maleimide group slowly hydrolyses when in contact with water, it was essential to proceed for the synthesis of immunoliposomes without unnecessary delay. The efficiency of coupling was not affected by mAb concentration using molar ratios of phospholipid/mAb from 100:1 to 400:1. An average of 10% of the antibody could be attached by a thioether bond to the liposome in most cases. Immunoliposomes were separated from unincorporated [^{3}H]daunomycin and free mAb and the external buffer was exchanged for 0.001 M PBS by a Sephadex CL-4B gel filtration chromatography (FIG. 3). [^{125}I]mAb OX26 eluted in two baseline-separated peaks at 12 and 29 ml, corresponding to liposome-conjugated mAb overlapping with the peak of [^{3}H]daunomycin containing liposomes and unbound mAb, respectively. The salt volume of the column was 35 ml. Analysis of elution profiles allowed the determination of efficiency of [^{3}H]daunomycin loading and conjugation of mAb. The conversion of μg of IgG per μmol of phospholipid to the number of mAb molecules conjugated per liposome was based on the assumption that a 100-nm liposome contains approximately 100,000 molecules of phospholipid (11).

Comparison of Free Daunomycin, Liposomes, Sterically Stabilized Liposomes, and Immunoliposomes. The disappearance of free daunomycin, liposomes, immunoliposomes, and sterically stabilized PEG-liposomes from the plasma compartment occurred in a biexponential manner (FIG. 4). From the data in FIG. 4, the distribution volume at steady state and the plasma clearance were calculated. There was a pronounced difference between these compounds. Free daunomycin and not PEG-conjugated liposomes containing daunomycin disappear rapidly from the circulation, with plasma clearances of 45 ± 7 and 13 ± 6 ml/min per kg, respectively (Table 1). The plasma clearance of the liposome was reduced 66-fold by PEG conjugation (Table 1). The 235-fold difference in plasma clearance between free daunomycin and daunomycin encapsulated within PEG-liposomes is indicative of adequate retention of the drug within liposomes. Coupling of 29 mAb $OX26_{29}$ per PEG-liposome partially reversed the effect of PEG conjugation and resulted in a 5-fold increase in plasma clearance (Table 1). Analysis of brain tissue revealed a permeability surface area (*PS*) product of 1.6μl/min per gram of free daunomycin (FIGS. 6-9), a value comparable to morphine-6-glucuronide (31).

However, the area under the plasma curve (*AUC*) of daunomycin is very small, resulting in poor brain tissue accumulation (reflected by its low %ID/g value) of this substance (FIGS. 8 and 9). The BBB *PS* product of the [^{3}H]daunomycin-containing liposomes was decreased 8-fold, whereas the plasma AUC was increased 4-fold compared with free daunomycin, and these offsetting effects resulted in no change in brain drug delivery (FIGS. 6-9). The use of PEG-conjugated liposomes reduced the BBB *PS* value to zero: therefore, no brain uptake of the PEG-liposomes was observed, despite the marked increase in plasma *AUC* (FIG. 8). Conversely, the use of PEG-conjugated OX26 immunoliposomes increased the BBB *PS* product, relative to PEG-liposomes, and a brain uptake of 0.03 %ID/g at 60 minutes was observed (FIGS. 6-9).

Titration of OX26. Titration of the amount of OX26 conjugated per liposome revealed an increase in plasma clearance and a decrease in the systemic volume of distribution of immunoliposomes at higher OX26 concentrations (FIG. 5 and Table 1). The PEG-liposomes are designated as $OX26_0$ immunoliposomes in FIG. 4. Highest *PS* product values and brain tissue accumulation were observed for $OX26_{29}$ immunoliposomes (FIGS. 10-13). At higher OX26 densities on the liposome, a saturation effect was observed resulting in reduced V_D, *PS* product, and brain tissue accumulation of $OX26_{197}$ immunoliposomes.

Twenty-four-Hour Pharmacokinetics and Tissue Distribution. To determine if immunoliposomes accumulate in brain tissue over time, 1-h, 6-h, and 24-h brain uptake experiments were performed using $OX26_{30}$ immunoliposomes, and the brain V_D was observed to increase over time (FIG. 14). This result indicates that immunoliposomes directed to the brain are trapped in the brain. Similar results were obtained from experiments using $OX26_{197}$ immunoliposomes (data not shown).

TABLE 1.

Systemic volume of distribution and plasma clearance of daunomycin, liposomes, and immunoliposomes

	Systemic volume of distribution,* ml/kg	Plasma clearance* ml/min/kg
Daunomycin	1400 ± 253	44.7 ± 6.8
Liposomes	290 ± 69	12.6 ± 6.3
PEG-liposomes	34 ± 4	0.19 ± 0.01
$OX26_3$	30 ± 3	0.22 ± 0.03
$OX26_{21}$	55 ± 2	1.20 ± 0.06
$OX26_{29}$	58 ± 9	0.91 ± 0.11
$OX26_{197}$	59 ± 3	0.67 ± 0.05
$OX26_{197}$ plus 1 mg of OX26	50 ± 4	0.39 ± 0.04†
20 Mouse IgG_{2a}	39 ± 4‡	0.37 ± 0.04‡

*Values represent means ± SEM of n = 3 experiments. All immunoliposomes are PEG-conjugated.

†Statistically significant difference by Student's t test (P = 0.012) when compared to corresponding value of $OX26_{197}$ immunoliposomes.

‡Statistically significant difference by Student's t test (P < 0.015) when compared to corresponding value of $OX26_{21}$ immunoliposomes.

Coinjection of OX26 and Use of Nonspecific Control IgG Immunoliposomes. In order to determine if brain delivery of immunoliposomes was mediated by the mAb OX26, two series of control experiments were performed. First, $OX26_{197}$ immunoliposomes were co-injected with 1 mg OX26. Comparison of plasma pharmacokinetics revealed a statistically significant difference (Student's t-test, $p < 0.012$) between $OX26_{197}$ and coinjection of 1 mg OX26 with $OX26_{197}$ with respect to plasma clearance (Table 1) and steady state area under the plasma concentration curve (197.2 ± 5.1 vs. 460.5 ± 27.8% ID·min/ml, means ± SEM, n = 3). The coinjection of free OX26 reduced the plasma clearance of the PEG-conjugated OX26 immunoliposome to a value equal to the plasma clearance of the PEG-conjugated IgG_{2a} immunoliposome (Table 1). Second, mouse IgG_{2a} (i.e. the OX26 isotype) was coupled to PEG-liposomes (20 mouse IgG per liposome) and pharmacokinetic parameters and tissue distribution were compared to PEG-conjugated $OX26_{21}$ immunoliposomes. The plasma clearance of the PEG-conjugated IgG_{2a} immunoliposomes was reduced 70% to a value identical to the clearance of the OX26 immunoliposome under saturating conditions (Table 1).

There was no brain uptake of the PEG-conjugated IgG_{2a} immunoliposomes, as the brain V_D, 7.4 ± 0.5 µl/g (n = 3), was not different from the brain V_D of PEG-liposomes, 7.5 ± 0.3 µl/g (n = 3).

Endocytosis by Transferrin Receptor Positive Cells. The endocytosis of the OX26 pegylated immunoliposome by transferrin receptor positive cells was demonstrated using RG2 cells in tissue culture. RG2 rat glioma cells expressing the transferrin receptor was seeded at a density of 250,000 cells/cm^2 on circular microscope glass cover slips. Cell culture medium was HAM F-12 containing 10% calf serum (Gibco BRL, Grand Island, NY). After four days when cells were 80% confluent, cell culture medium was exchanged for 0.001 M PBS, pH 7.4: HAM F-12 = 1 (v/v). Fluorescent immunoliposomes were added (20 µg/ml MAb) and the cells were incubated for 2 hours at 37°C. Cells were washed, fixed for 20 minutes at 4°C using 4% paraformaldehyde and were mounted in 5% n-propyl gallate in 100% glycerol. Confocal microscopy was done using a Leitz/Leica Fluovert FU inverted fluorescence microscope with Leica CLSM adapter. In order to determine if immunoliposomes can potentially penetrate cells by means of receptor mediated endocytosis, cultured RG2 rat glioma cells expressing the transferrin receptor were incubated with fluorescent $OX26_{24}$ immunoliposomes. Analysis by confocal microscopy revealed accumulation of fluorescence in RG2 cells exposed to $OX26_{24}$ immunoliposomes. High magnification of the cell revealed intracellular fluorescence in particles presumed to be endosomes. Cell staining was not observed in control experiments using fluorescent IgG_{2a} isotype control immunoliposomes.

Uptake of OX26 By Rat Brain Capillaries. Fluorescein-labeled immunoliposomes, conjugated with either the OX26 MAb or the mouse IgG_{2a} isotype control, were incubated with freshly isolated rat brain capillaries. Stained capillaries were not fixed but directly transferred to a glass slide and analyzed by confocal microscopy. Incubations with OX26 immunoliposomes resulted in staining both luminal and abluminal membranes of the brain capillary endothelium. In contrast, immunoliposomes conjugated with the mouse IgG_{2a} gave no noticeable staining of the capillary endothelial membranes. Immunostaining of nonfixed capillaries allowed for the discrimination between the luminal and basolateral membrane domains in the endothelium. Computer-aided reconstruction of consecutive optical

sections through the brain capillary revealed the intactness of the three-dimensional structure of the microvessel, as the following structures were visible: the interior lumen, the luminal membrane of the endothelium, the endothelial cytoplasm, and the abluminal endothelial membrane.

In other experiments, the uptake of unconjugated OX26 MAb was investigated in parallel with rhodamine-phosphatidylethanolamine (PE), used as a non-specific fluorescent marker for membranes. The uptake of the OX26 MAb by isolated rat brain capillaries was demonstrated, and visualization of the distribution of the OX26 MAb with a fluorescein-labeled secondary antibody, revealed a punctate immunostaining pattern consistent with uptake of the OX26 MAb into endocytotic vesicles of the rat brain capillary endothelium. Overlay of the fluorescent signal from rhodamine-PE and the transferrin receptor/OX26 signal reveal a localization of the transferrin receptor within intracellular endosomes.

The above examples are described and discussed in further detail in Reference Nos. 40-42.

Having thus described exemplary embodiments of the present invention, it should be noted by those skilled in the art that the within disclosures are exemplary only and that various other alternatives, adaptations, and modifications may be made within the scope of the present invention. Accordingly, the present invention is not limited to the specific embodiments as illustrated herein, but is only limited by the following claims.

BIBLIOGRAPHY

1. Aragnol, D. & Leserman, L.D. 9186) *Proc. Natl. Acad. Sci. USA* **83**, 2699-2703.

2. Derksen, J.T., Morselt, H.W. & Scherphof, G.L. (1988) *Biochim. Biophys. Acta* **971**, 127-136.

3. Gabizon, A. & Papahadjopoulos, D. (1992) *Biochim. Biophys. Acta* **1103**, 94-100.

4. Papahadjopoulos, D., Allen, T.M., Gabizon, A., Mayhew, E., Matthay, K., Huang, S.K., Lee, K.D., Woodle, M.C., Lasic, D.D., Redemann, C. & Martin, F.J. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 11460-11464.

5. Allen, T.M. (1994) *Trends Pharmacol. Sci.* **15**, 215-220.

6. Moghimi, S.M. & Patel, H.M. (1992) *Biochim. Biophys. Acta* **1135**, 269-274.

7. Klibanov, A.L., Maruyama, K., Beckerieg, A.M., Torchilin, V.P. & Huang, L. (1991) *Biochim. Biophys. Acta* **1062**, 142-148.

8. Torchilin, V.P. (1994) *ImmunoMethods* **4**, 244-258.

9. Lee, R.J. & Low, P.S. (1994) *J. Biol. Chem.* **269**, 3198-3204.

10. Allen, T.M., Brandeis, E., Hansen, C.B., Kao, G.Y. & Zalipsky, S. (1995) *Biochim. Biophys. Acta* **1237**, 99-108.

11. Hansen, C.B., Kao, G.Y., Moase, E.H., Zalipsky, S. & Allen, T.M. (1995) *Biochim. Biophys. Acta* **1239**, 133-144.

12. Maruyama, K. Takizawa, T., Yuda, T., Kennel, S.J., Huang, L. & Iwatsuru, M. (1995) *Biochim. Biophys. Acta* **1234**, 74-80.

13. Shahinian, S. & Silvius, J.R. (1995) *Biochim. Biophys. Acta* **1239**, 157-167.

14. Schackert, G., Fan, D., Nayar, R. & Fidler, L.J. (1989) *Sel. Cancer Ther.* **5**, 73-79.

15. Gennuso, R., Spigelman, M.K., Chinol, M., Zappulla, R.A., Nieves, J., Vallabhajosula, S., Alberto, P.P., Goldsmith, S.J. & Holland, J.F. (1993) *Cancer Invest.* **11**, 118-128.

16. Sakamoto, A. & Ido, T. (1993) *Brain Res.* **629**, 171-175.

17. Pardridge, W.M. (1995) *Adv. Drug Delivery Rev.* **15**, 109-146.

18. Bickel, U., Kang, Y.S., Yoshikawa, T. & Pardridge, W.M. (1994) *J. Hostochem. Cytochem.* **42**, 1493-1497.

19. Jefferies, W.A., Brandon, M.R., Williams, A.F. & Hunt, S.V. (1985) *Immunology* **54**, 333-341.

20. Jefferies, W.A., Brandon, M.R., Hunt, S.V., Williams, A.F., Gatter, K.C. & Mason, D.Y. (1984) *Nature (London)* **312**, 162-163.

21. Pardridge, W.M., Eisenberg, J. & Yang, J. (1987) *Metabolism* **36**, 892-895.

22. Kang, Y.S. & Pardridge, W.M. (1994) *J. Pharmacol. Exp. Ther.* **269**, 344-350.

23. Yoshikawa, T. & Pardridge, W.M. (1992) *J. Pharmacol. Exp. Ther.* **263**, 897-903.

24. Triguero, D., Buciak, J.B., Yang, J. & Pardridge, W.M. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 4761-4765.

25. Marsh, J.W. (1988) *J. Biol. Chem.* **263**, 15993-15999.

26. Riddles, P.W., Blakeley, R.L. & Zerner, B. (1979) *Anal. Biochem.* **94**, 75-81.

27. Mayer, L.D., Bally, M.B. & Cullis, P.R. (1986) *Biochem. Biophys. Acta* **857**, 123-126.

28. Heath, T.D., Macher, B.A. & Papahadjopoulos, D. (1981) *Biochem. Biophys. Acta* **640**, 66-81.

29. Kang, Y.S., Bickel, U. & Pardridge, W.M. 91994) *Drug. Metab. Dispos.* **22**, 99-105.

30. Pardridge, W.M., Boado, R.J. & Kang, Y.S. (1995) *Proc. Natl. Acad. Sci. USA* **92**, 5592-5596.

31. Bickel, U., Schumacher, O., Kang, Y.S. & Voigt, K. (1996) *J. Pharmacol. Exp. Ther.*, **278**, 107-113.

32. Pardridge, W.M., Buciak, J.L. & Friden, P.M. (1991) *J. Pharmacol. Exp. Ther.* **259**, 66-70.

33. Skarlatos, S., Yoshikawa, T. & Pardridge, W.M. (1995) *Brain Res.* **683**, 164-171.

34. Martin, F.J. & Papahadjopoulos, D. (1982) *J. Biol. Chem.* **257**, 286-288.

35. Heath, T.D. & Martin, F.J. (1986) *Chem. Phys. Lipids* **40**, 347-358.

36. Mayer, L.D., Tai, L.C., Ko, D.S., Masin, D., Ginsberg, R.S., Cullis, P.R. & Bally, M.B. (1989) *Cancer Res.* **49**, 5922-5930.

37. Allen, T.M. & Martin, F.J., U.S. Patent No. 5,527,528.

38. Pardridge, W.M., U.S. Patent No. 4,801,575.

39. Wu, D., Yang, J. & Pardridge, W.M. (1997) *J. Clin. Invest.* **100**, 1804-1812.

40. Huwyler, J., Wu, D. & Pardridge, W.M. (1996) *Proc. Natl. Acad. Sci. USA* **93**, 14164-14169.

41. Huwyler, J., Yang, J. & Pardridge, W.M. (1997) *J. Pharmacol. Exp. Ther.* **282**, 1541-1546.

42. Huwyler, J. & Pardridge, W.M. (1998) *J. Neurochem.*, in press.

CLAIMS 23

What is claimed is:

1. A brain-specific liposome targeting vehicle for transporting neuropharmaceutical agents across the blood-brain barrier, said liposome targeting vehicle comprising:

a liposome comprising a vesicle which has an exterior surface and an interior surface, said interior surface defining a compartment for a neuropharmaceutical agent;

a ligand comprising a head portion and a tail portion, wherein said the head portion is attached to said exterior surface of said liposome;

a blood-barrier transport agent which is attached to the tail portion of said ligand; and

a neuropharmaceutical agent located within said liposome compartment.

2. A brain-specific liposome targeting vehicle according to claim **1** wherein said liposome has a diameter in the range of 40 microns to 100 microns.

3. A brain-specific liposome targeting vehicle according to claim **2** wherein said liposome is unilamelar.

4. A brain-specific liposome targeting vehicle according to claim **1** wherein said ligand is selected from the group consisting of polyethylene glycol, ganglioside, or glycerophosphatidylinositol.

5. A brain-specific liposome targeting vehicle according to claim **1** wherein said blood-barrier transport agent is selected from the group consisting of transportable peptides and antibodies.

6. A brain-specific liposome targeting vehicle according to claim **5** wherein said blood-barrier transport agent is a monoclonal antibody.

24

7. A brain-specific liposome targeting vehicle according to claim 6 wherein said ligand is polyethylene glycol.

8. A brain-specific liposome targeting vehicle according to claim 7 wherein said monoclonal antibody is selected from the group consisting of antibodies against the transferrin, inulin, IGF, leptin, or prolactin receptor.

9. A brain-specific liposome targeting vehicle according to claim 1 wherein said vehicle comprises a plurality of ligands attached to the surface of said liposome and wherein from about 10 to 40 blood-barrier transport agents are attached to said liposome via said ligands.

10. A brain-specific liposome targeting vehicle according to claim 9 wherein said blood-barrier transport agent is a monoclonal antibody.

11. A brain-specific liposome targeting vehicle according to claim 9 wherein the number of blood-barrier transport agents attached to said liposome is about 30.

12. A method for delivering a neuropharmaceutical agent into the brain of an animal by transcytosis across the blood-brain barrier, said method comprising the step of introducing into the bloodstream of said animal a brain-specific liposome targeting vehicle, said targeting vehicle comprising:

a liposome comprising a vesicle which has an exterior surface and an interior surface, said interior surface defining a compartment for neuropharmaceutical agents;

a ligand comprising a head portion and a tail portion, wherein said the head portion is attached to said exterior surface of said liposome;

a blood-barrier transport agent which is attached to the tail portion of said ligand; and

a neuropharmaceutical agent located within said liposome compartment.

13. A method according to claim **9** wherein said liposome has a diameter in the range of about 40 microns and 100 microns.

14. A method according to claim **10** wherein said liposome is unilamelar.

15. A method according to claim **9** wherein said ligand is selected from the group consisting of polyethylene glycol, ganglioside, or glycerophosphatidylinositol.

16. A method according to claim **9** wherein said blood-barrier transport agent is selected from the group consisting of transportable peptides and antibodies.

17. A method according to claim **13** wherein said blood-barrier transport agent is a monoclonal antibody.

18. A method according to claim **14** wherein said ligand is polyethylene glycol.

19. A method according to claim **15** wherein said monoclonal antibody is selected from the group consisting of antibodies against the transferrin, insulin, IGF, leptin, or prolactin receptor.

20. A method according to claim **12** wherein said vehicle comprises a plurality of ligands attached to the surface of said liposome and wherein from about 10 to 40 blood-barrier transport agents are attached to said liposome via said ligands.

21. A method according to claim **20** wherein said blood-barrier transport agent is a monoclonal antibody.

22. A method according to claim **21** wherein the number of blood-barrier transport agents attached to said liposome is about 30.

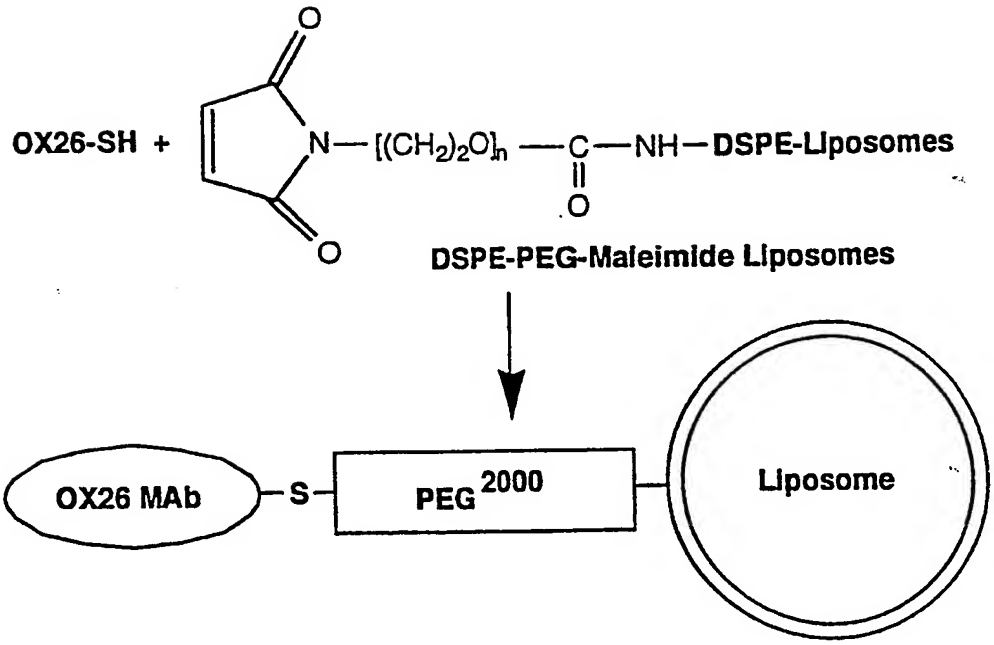
OX26-SH +
N—[(CH2)2O]n—C—NH—DSPE-Liposomes
O
O
O
DSPE-PEG-Maleimide Liposomes
OX26 MAb
S
PEG 2000
Liposome

FIG. 1

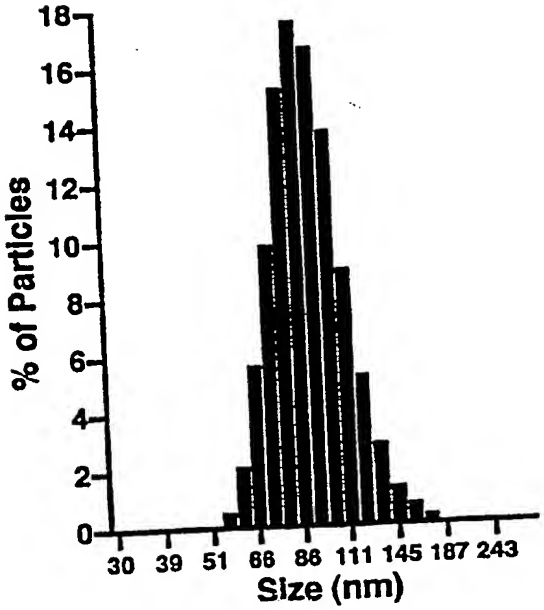
18
16
14
12
10
8
6
4
2
0
% of Particles
30 39 51 66 86 111 145 187 243
Size (nm)

FIG. 2

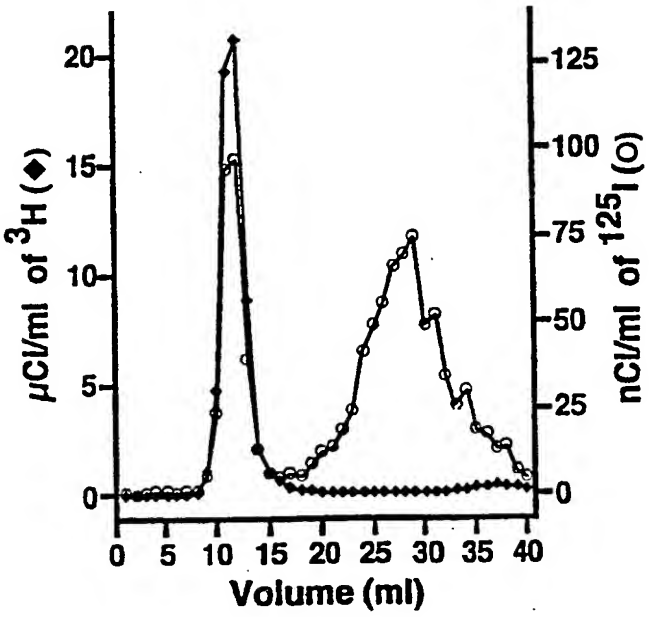
20
15
10
5
0
μCi/ml of ^{3}H (◆)
125
100
75
50
25
0
nCi/ml of ^{125}I (O)
0 5 10 15 20 25 30 35 40
Volume (ml)

FIG. 3

4/10

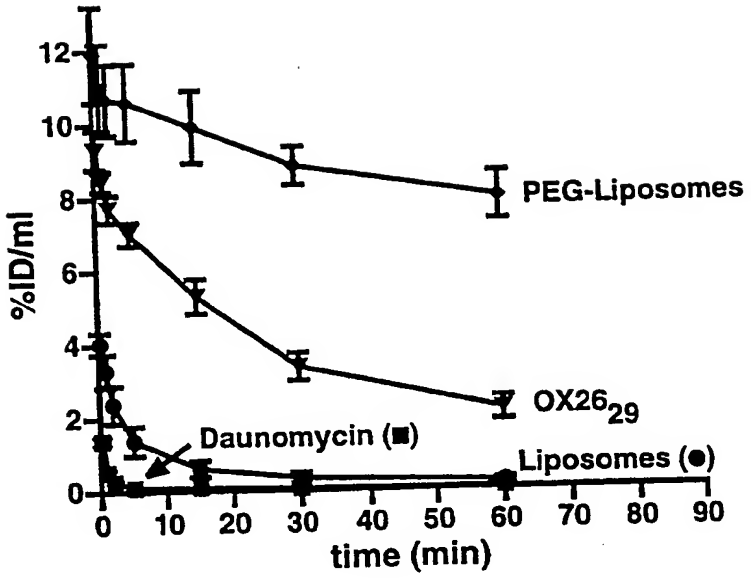
12
10
8
6
4
2
0
%ID/ml
0
10
20
30
40
50
60
70
80
90
time (min)
PEG-Liposomes
OX26$_{29}$
Daunomycin (■)
Liposomes (●)

FIG. 4

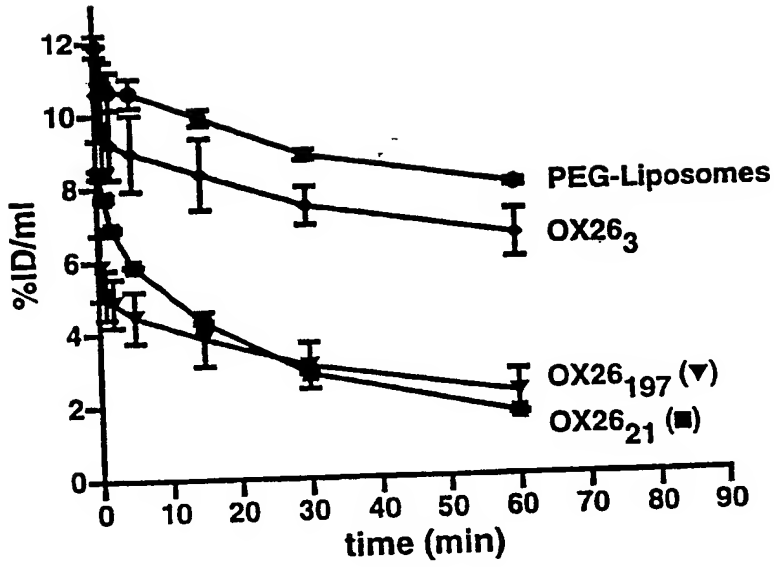
12
10
8
6
4
2
0
%ID/ml
0
10
20
30
40
50
60
70
80
90
time (min)
PEG-Liposomes
OX26$_3$
OX26$_{197}$ (▼)
OX26$_{21}$ (■)

FIG. 5

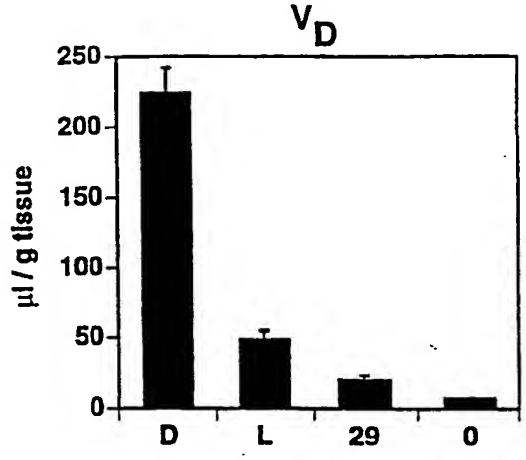
V_D
250
200
150
100
50
0
µl / g tissue
D
L
29
0

FIG. 6

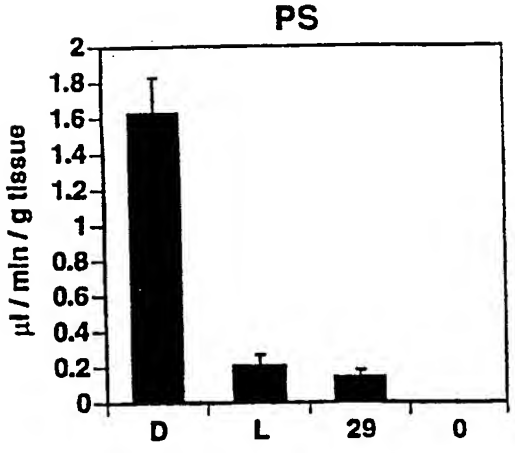
PS
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
µl / min / g tissue
D
L
29
0

FIG. 7

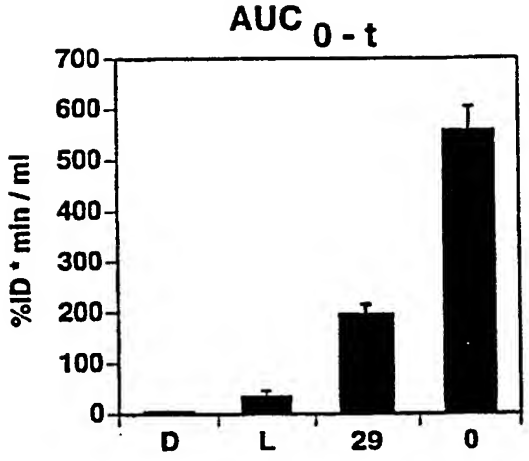
AUC 0 - t
%ID * min / ml
700
600
500
400
300
200
100
0
D
L
29
0

FIG. 8

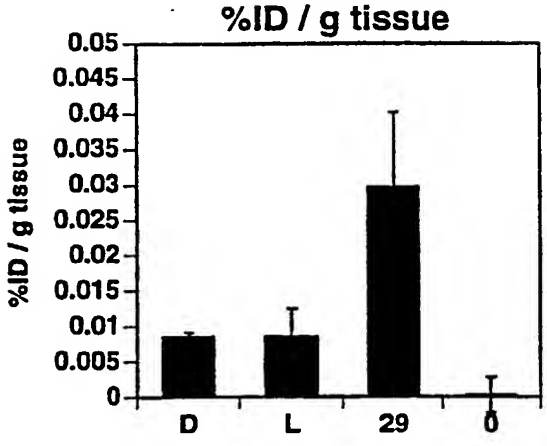
%ID / g tissue
%ID / g tissue
0.05
0.045
0.04
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
D
L
29
0

FIG. 9

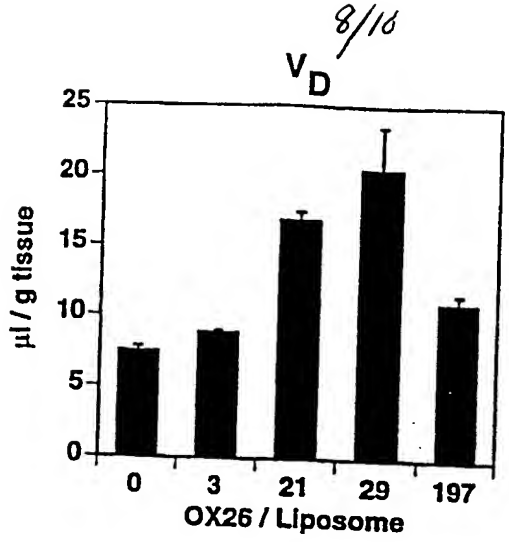
V_D
25
20
15
10
5
0
µl / g tissue
0
3
21
29
197
OX26 / Liposome

FIG. 10

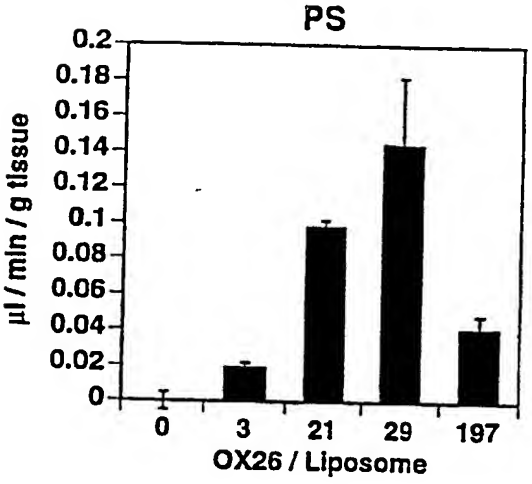
PS
0.2
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
µl / min / g tissue
0
3
21
29
197
OX26 / Liposome

FIG. 11

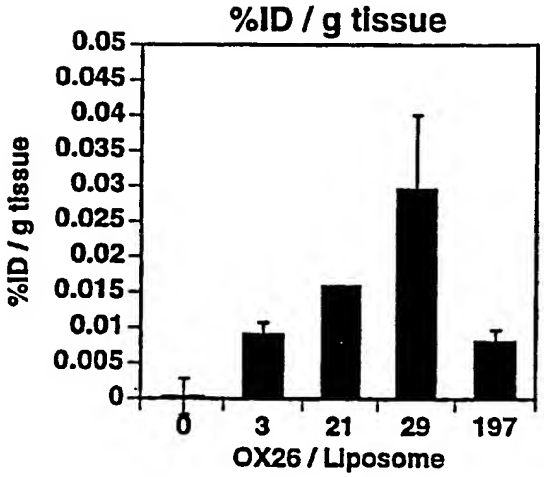
%ID / g tissue
%ID / g tissue
0.05
0.045
0.04
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
0
3
21
29
197
OX26 / Liposome

FIG. 12

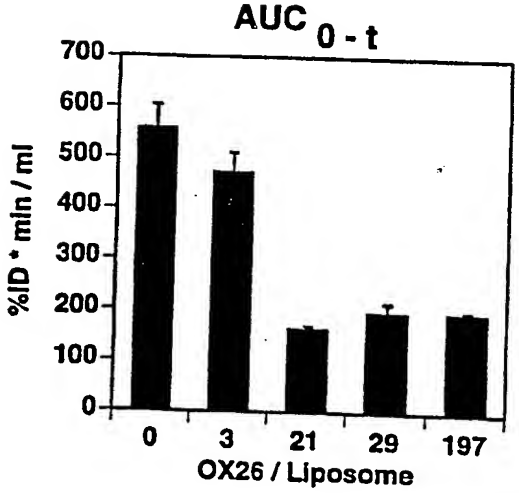
AUC 0 - t
%ID * min / ml
700
600
500
400
300
200
100
0
0
3
21
29
197
OX26 / Liposome

FIG. 13

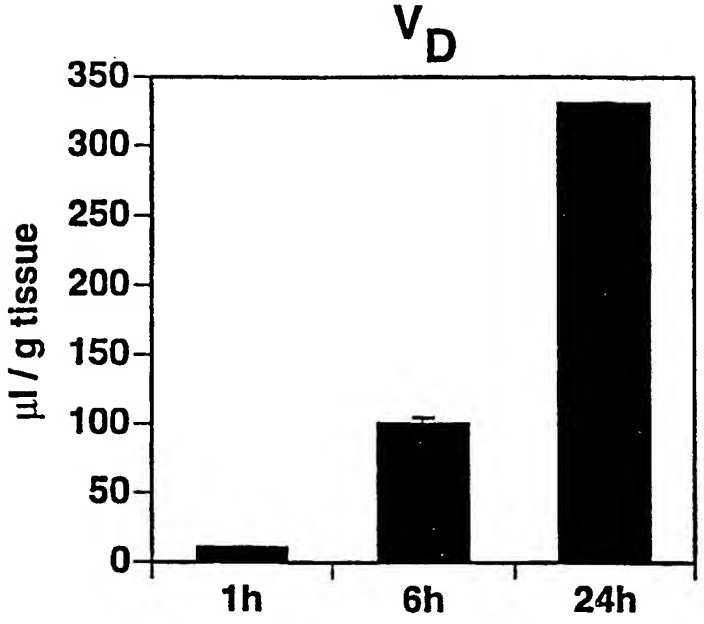

FIG. 14

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/21352

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 9/127, 9/133
US CL :424/450; 436/829

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/450; 436/829

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS:
search terms: blood brain barrier, liposomes, antibodies, PEG.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,527,528 A (ALLEN et al) 18 June 1996, abstract, figures, column 2, line32-39, column 6, line 11 through column 11, line 30, example 1 and claims.	1-7 & 12-18
Y	US 5,576,018 A (KIM et al) 19 November 1996, abstract, column 3, line 30 through column 8, line 10.	1-22
Y	US 5,543,390 A (YATVIN et al) 06 August 1996, abstract and column 17, lines 1-19.	1-22

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 04 FEBRUARY 1998	Date of mailing of the international search report 04 MAR 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer G. S. KISHORE Telephone No. (703) 308-2351

Form PCT/ISA/210 (second sheet)(July 1992)★